FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
08213/007001	09/423546

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/09988	15 May 1998 (15.05.1998)	16 May 1997 (16.05.1997)

TITLE OF INVENTION

VACCINE AGAINST LIPOPOLYSACCHARIDE CORE

APPLICANT(S) FOR DO/EO/US

Elliot BENNETT-GUERRERO; George Robin BARCLAY; Ian Raymond POXTON; Thomas James MCINTOSH; David Scott SNYDER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☐ Other items or information:

"Express Mail" label number: EL445347140US
Date of Deposit : 12 November 1999
I hereby certify that under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office To Addressee" with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, DC 20231.

Samantha Bell

Samantha Bell

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/423546	PCT/US98/09988	08213-007001

20. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS	PTO USE ONLY
☐	Search Report has been prepared by the EPO or JPO	$840.00	
☒	International preliminary examination fee paid to USPTO (37 CFR 1.482)	$670.00	
☐	No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	$760.00	
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO	$970.00	
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$96.00	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	$670.00	
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	$0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	61 - 20 =	41	x $22.00	$902.00	
Independent claims	4 - 3 =	1	x $82.00	$82.00	
Multiple Dependent Claims **(check if applicable)** .			☒	$260.00	
	TOTAL OF ABOVE CALCULATIONS		=	$1,914.00	
	Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)** .		☐	$0.00	
	SUBTOTAL		=	$1,914.00	
	Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).		+	$0.00	
	TOTAL NATIONAL FEE		=	$1,914.00	
	Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**		☐	$0.00	
	TOTAL FEES ENCLOSED		=	$1,914.00	
				Amount to be: refunded	$
				charged	$

☒ A check in the amount of **$1,914.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **06-1050** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO.

John W. Freeman
Fish & Richardson PC
225 Franklin Street
Boston, Massachusetts 02110

John W. Freeman
SIGNATURE

John W. Freeman
NAME

29,066
REGISTRATION NUMBER

11/12/99
DATE

Attorney's Docket No.: 08213-007001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Elliott Bennett-Guerrero et al. Art Unit : Unknown
Serial No. : Examiner : Unknown
Filed :
Title : VACCINE AGAINST LIPOPOLYSACCHARIDE CORE

Assistant Commissioner for Patents
Washington, D.C. 20231

International Patent Application No.: PCT/US98/09988

International Filing Date: 15 May 1998

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the Specification:

Page 1, before the first line, please insert the following paragraph: --This is a continuation of International Patent Application No. PCT/US98/09988, with an international filing date of May 15, 1998, which is a continuation of USSN 60/046,680, now abandoned.--

In the Claims:

Delete claims 1-43, and add the following new claims:

-- 44. A method of reducing adverse effects of endotoxin in a warm-blooded animal, which comprises administering to the warm-bloooded animal an effective amount of a composition comprising rough, complete-core lipopolysaccharide (LPS) antigen of a gram negative bacterium.

CERTIFICATE OF MAILING BY EXPRESS MAIL

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12 November 1999
Date of Deposit

Samantha Bell
Signature

Samantha Bell
Typed or Printed Name of Person Signing Certificate

45. The method of claim 44 in which the composition comprises rough, complete-core lipopolysaccharide (LPS) antigen of at least two of the following gram negative bacterium: *E. coli*; *Pseudomonas; Klebsiella; Salmonella and Bacteroides.*

46. The method of claim 45 in which the composition comprises rough, complete-core lipopolysaccharide (LPS) antigen of at least *E. coli*; *Pseudomonas; and Bacteroides.*

47. The method of claim 44 in which the composition comprises killed whole cells.

48. The method of claim 47 in which the composition comprises killed whole E. coli K12, containing rough complete-core LPS antigen.

49. The method of claim 45 in which the composition comprises a cocktail of killed Ra chemotype whole-cell mutants of at least three of the following species of gram-negative bacteria: *E. coli* K12, *E. coli* R1, *Bacteroides fragilis*, *Pseudomonas aeruginosa.*

50. The method of claim 49 in which the composition comprises killed whole-cell Ra LPS mutants from each of the following species of gram-negative bacteria: *E. coli* K12, *E. coli* R1, *Bacteroides fragilis, Pseudomonas aeruginosa.*

51. The method of claim 44 in which the composition comprises purified detoxified Ra LPS conjugated to protein.

52. The method of claim 51 in which the Ra LPS conjugate is conjugated *E. coli* K12 Ra LPS.

53. The method of claim 51 in which the composition comprises a cocktail of Ra LPS from multiple species of gram-negative bacteria, conjugated to a protein.

54. The method of claim 53 in which the composition comprises conjugates of Ra LPSs from at least three of the following species of gram-negative bacteria: *E. coli* K12, *E. coli* R1, *Bacteroides* fragilis, *Pseudomonas aeruginosa.*

55. The method of claim 53 in which the composition comprises Ra LPS incorporated in a liposome.

56. The method of claim 55 in which the composition comprises E. coli K12 Ra LPS in a liposome.

57. The method of claim 55 in which the composition comprises a cocktail of Ra LPSs from multiple species of gram-negative bacteria incorporated in liposomes.

58. The method of claim 57 in which the cocktail comprises Ra LPSs from at least three of the following species of gram-negative bacteria: *E. coli* K12, *E. coli* R1, *Bacteroides fragilis*, and *Pseudomonas aeruginosa.*

59. The method of claim 44 in which the composition comprises rough, complete-core lipopolysaccharide (LPS) antigen of *E. coli* K12.

60. The method of claim 59 in which the composition further comprises rough, complete-core lipopolysaccharide (LPS) antigen of a second bacteria other than *E. coli* K12.

61. The method of claim 60 in which the animal is a mammal.

62. The method of claim 61 in which the animal is a human patient.

63. The method of claim 59 in which the composition comprises LPS of an Ra rough *E. coli* K12.

64. The method of claim 60 in which the second bacterium is an *E. coli* or a *Salmonella* bacterium.

65. The method of claim 60 in which the second bacteria is a *Bacteroide*s.

66. The method of claim 60 in which the composition comprises complete-core, rough, LPS antigen from a third Gram-negative bacterium different from the first and from the second Gram-negative bacterium.

67. The method of claim 66 in which the composition comprises complete-core, rough, LPS antigen from a fourth Gram-negative bacterium different from each of the first, the second, and the third Gram-negative bacteria.

68. The method of claim 59 in which the second Gram-negative bacterium is *E. coli* R1.

69. The method of claim 59 in which the second Gram-negative bacterium is a *Salmonella* bacterium.

70. The method of claim 66 in which the second bacterium is a *Klebsiella* and third bacterium is a *Pseudomonad.*

71. The method of claim 67 in which the second bacterium is a *Klebsiella*, the third bacterium is a Pseudomonad, and the fourth bacterium is a *Bacteroides*.

72. The method of claim 64 or claim 69 in which the *Salmonella* bacterium is *Salmonella minnesota* .

73. The method of claim 67 in which complete core antigen from each of the four bacteria is present in generally equal amounts by weight.

74. The method of claim 66 in which the composition comprises LPS antigens from at least two different Gram-negative bacterial strains of the same species.

75. The method of claim 59 in which the antigen causes the patient to produce an antibody that binds to an epitope in the core region of the LPS of at least one Gram-negative bacterial strain whose LPS is not part of the composition.

76. The method of claim 75 in which the patient's antibody binds to the LPS of at least one smooth Gram negative bacterial strain.

77. The method of claim 75 in which the composition comprises the antigen in a liposome.

78. The method of claim 77 in which the ratio (weight:weight) of lipid in the liposome to the LPS antigen is between 1:1 and 5000:1.

79. The method of claim 77 in which the ratio (weight:weight) is between 10:1 and 1000:1.

80. The method of claim 77 in which the liposome comprises a component selected from the group consisting of: phospholipid, cholesterol, positively charged compounds, negatively charged compounds, amphipathic compounds.

81. The method of claim 77 in which the liposome is a multilamellar type liposome (MLV).

82. The method of claim 77 in which LPS in the acid salt form is incorporated into the liposome.

83. The method of claim 77 in which the liposome is a small or large unilamellar liposome (SUVs and LUVs).

84. The method of claim 59 in which the composition is administered intramuscularly, intravenously, subcutaneously, intraperitonealy, via the respiratory tract, or via the gastrointestinal tract.

85. The method of claim 59 in which the dose of antigen is over 0.01 ng per kilogram of patient body weight.

86. The method of claim 85 in which the dose is less than 1000ng per kilogram of patient body weight.

87. The method of claim 85 in which the dose is less than 100 micrograms per kilogram of patient body weight.

88. The method of claim 59 in which the composition is administered in multiple doses, the first of which is administered at least 2 days prior to potential endotoxin exposure.

89. The method of claim 59 in which the antigen is present in a killed bacterium.

90. The method of claim 59 in which the antigen is separated from the bacterium.

91. The method of claim 59 in which the antigen is chemically detoxified.

92. The method of claim 59 or claim 90 in which the bacterium is genetically engineered.

93. The method of claim 59 in which the composition further comprises an adjuvant.

94. The method of claim 93 in which the adjuvant is alum.

95. A vaccine composition for reducing the adverse effects of endotoxemia in a human patient which comprises an effective amount of a composition comprising purified complete core rough lipopolysaccharide antigen of *E. coli* K12, said composition further comprising liposomes which contain the antigen.

96. A method of reducing adverse effects of endotoxin in a warm-blooded animal, which method comprises administering to the warm-blooded animal an effective amount of a composition comprising rough lipopolysaccharide (LPS) antigen of a Gram-negative bacterium, said LPS antigen comprising the component of an *E. coli* Rb chemotype LPS, or the equivalent thereof in another species.

97. A method of quantitating lipopolysaccharide incorporated into liposomes by performing periodic acid/Schiff base staining.

98. The method of claim 97 in which the test is performed on a vaccine lot intended for clinical use.

99. A method of reducing adverse effects of endotoxin in a warm-blooded animal, which method comprises administering to the warm-blooded animal an effective amount of antibody produced by immunization with a composition accoring to claim 44 or claim 59.

100. The method of claim 99 in which the antibody comprises a substantial percentage of IgM antibody.

REMARKS

The specification is amended to recite claimed benefit under 35 USC 120 from International Patent Application PCT/US98/09988 and its US application.

Applicant submits that no new matters has been introduced.

Claims 44-100 are now pending. Prompt examination of the present application, as amended, is respectfully requested.

Please charge any fees or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 11/9/99

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ATTORNEY DOCKET NO. 08213-007001

Applicant or Patentee: Elliott Bennett-Guerrero et al.
Serial or Patent No.: 09/423,546
Filed or Issued: November 12, 1999
For: VACCINE AGAINST LIPOPOLYSACCHARIDE CORE

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c)) — SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[X] an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Small Business Concern: Medical Defense Technologies, Inc.
Address of Small Business Concern: ~~175 East 96th Street #4G, New York, NY 10128~~ 95 Christopher Street, #136 NY NY 10014

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled VACCINE AGAINST LIPOPOLYSACCHARIDE CORE by inventor(s) IAN RAYMOND FOXTON, GEORGE ROBIN BARCLAY, THOMAS JAMES MCINTOSH, DR. ELLIOTT BENNETT-GUERRERO AND DAVID SCOTT SNYDER described in:

[ ] the specification filed herewith.
[X] application serial no. 09/423,546, filed November 12, 1999.
[ ] patent no. _, issued _.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name: _____
Address: _____

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status when any new rule 53 application is filed or prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent on which this verified statement is directed.

Name:	Elliott Bennett-Guerrero
Title:	President
Address:	95 Christopher Street, #136 NY NY 10014

Signature: _____ **Date:** 1-12-2000

VACCINE AGAINST LIPOPOLYSACCHARIDE CORE

Cross-reference to related application

This application is a conversion of our prior U.S. provisional application, U.S. 60/046,680, filed May 16, 1997, which is hereby incorporated by reference.

Field of the Invention

This invention is in the general field of reducing the adverse effects of endotoxin from Gram-negative bacteria.

Background of the Invention

Endotoxin (also called lipopolysaccharide [LPS]) is thought to exert many of its toxic effects following its entry into the bloodstream. The presence of endotoxin in the blood, endotoxemia, can occur in various situations, e.g., during periods of stress. For example, endotoxemia can occur in patients undergoing certain types of surgery, anti-cancer chemotherapy, radiation therapy, and immunosuppressive treatment, and it can also occur in patients suffering from various trauma, burns, or wounds. It occurs as well in military, police, and fire-fighting personnel as well as in endurance athletes, horses, and in livestock. It can also occur after immunosuppressive treatment, and in patients with sepsis or septic shock as well as in those suffering from stress or trauma as discussed above.

One way that endotoxin may reach the blood is from the patient's intestine because the intestine loses its ability to contain LPS during periods of infection, stress, or trauma. Normally, intestinal flora contain a large amount of endotoxin from Gram-negative microorganisms. It is estimated that the average human colon contains 25 billion nanograms of endotoxin, which is an enormous quantity when one considers that endotoxin concentrations on the order of 10^2 are toxic to humans.

The leakage of live bacterial cells into the bloodstream can result in infection as the bacteria multiply. Many of the bacteria in the intestine are dead, and endotoxin contained within cell membrane fragments of dead bacteria can also enter the bloodstream. In this case infection per se does not develop. Instead, endotoxin from dead bacteria in the blood is thought to initiate a systemic inflammatory response by activating macrophages which release tumor necrosis factor and various interleukins. Endotoxin exposure and the resulting systemic inflammatory response can cause damage to body organs, including the lungs, kidneys, heart, blood vessels, gastrointestinal tract, blood/coagulation system, and nervous system. This proinflammatory response can be severe, causing organs to fail, sometimes resulting in death.

LPS is thought to be a major causative agent of septic shock. It is increasingly recognized that less severe forms of this systemic inflammation cause organ dysfunction as opposed to organ failure. In its mildest form, endotoxemia can cause fever, nausea, and malaise, common symptoms of patients following surgery or patients who are hospitalized for other reasons, and the symptoms even occur, for example in athletes following strenuous activity.

Greater exposure of the host to endotoxin or a greater susceptibility to its effects can result in a larger inflammatory response. For example, many post surgical patients develop pulmonary dysfunction requiring supplemental oxygen. They may also develop hematologic or renal complications. These complications often do not lead to death but instead cause suffering and increase hospital length of stay and thus health care costs. It is estimated that at least 10% of the 28 million United States surgical patients may develop systemic

inflammation and possible complications as a result of exposure to endotoxin from Gram-negative microorganisms. See generally, Bennett-Guerrero et al., Crit Care Med, 25:A112, 1997 and Bennett-Guerrero et al., J. Am. Med. Ass. 277:646-650, 1997 discussing certain types of surgical patients at risk.

Among the bacteria and their respective endotoxins which are thought to commonly cause complications are *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeuruginosa, Proteus spp., Enterobacter spp., Salmonella spp., Serratia spp, and Shigella spp.* These bacteria are Gram-negative bacteria, a class characterized by a specific type of outer membrane which compromises a lipopolysaccharide (LPS) as a major constituent. Although the LPS constituent varies from one bacterial species to another, it may be generally described with reference to Fig. 1 as consisting of three structural regions: a) Lipid A; b) core; and c) O-polysaccharide outer region. The lipid region of Lipid A is embedded in the outer leaflet of the outer membrane. The oligosaccharide core region is positioned between Lipid A and the O-polysaccharide outer region. Lipid A has the same basic structure in practically all gram negative bacteria and is the main endotoxic determinant. The LPS core region shows a high degree of similarity among bacterial genera. It usually consists of a limited number of sugars. For example, the inner core region is constituted of heptose and 3-deoxy-D-*manno*-2-octulosonate (KDO) residues, while the outer core region comprises galactose, glucose, or N-acetyl-D-glucosamine residues displayed in various manners depending upon the strain. The O-polysaccharide outer region (also called O-specific antigen or O-specific side chain) is highly variable and is composed of one or more oligosaccharide repeating units characteristic of the serotype.

The presence of the O-polysaccharide side chain confers a smooth aspect to a culture of a wild type bacterium, and, for this reason, wild type bacteria with polysaccharide side chain are usually referred to as smooth bacteria in contrast with mutant cultures which show a rough aspect because they lack the O-polysaccharide side chain and (in some cases) part of the core region. For example, the different chemotypes of rough mutants from Salmonella are conventionally designated by the terms Ra, Rb, Rc, Rd, and Re.

As seen from Fig. 2, the LPS of each type comprises the lipid A structure. The Ra chemotype is characterized by a complete core region, the Rb chemotype is characterized by the absence of N-acetyl-D-glucosamine residues, the Rc chemotype is characterized by the absence of N-acetyl-D-glucosamine and galactose residues, the Rd chemotype is characterized by the absence of any residues constituting the outer core, and the Re chemotype is characterized by the sole KDO region attached to lipid A.

Fig. 3 is a diagrammatic representation of the five known complete core chemotypes of *E. coli* as well as the one known complete core chemotype of all Salmonella species.

Not all LPS molecules on the surface of a given cell or in a homogeneous population of cells have the same number of oligosaccharide side chains. For example, a single cell from a population of smooth strain bacteria may include some rough forms of LPS, i.e., LPS that is not substituted with any polysaccharide side chains.

Various treatments for the toxic effect of LPS have been proposed or tried. One of a mammal's defenses against endotoxemia is the presence of antibodies in the blood which can bind to and neutralize blood borne endotoxin, and immunologic methods have been proposed as

an alternative or additional treatment to antibiotic therapy to prevent or control such infections or to reduce the toxic effect of endotoxin. For example, conventional polyclonal antisera and hyperimmune sera have been used in an attempt to bolster the native defenses of patients against the adverse effects of bacteria, presumably by enhancing opsonization and phagocytosis of the bacterial cells or by neutralization of the biological activity of LPS. However, the effectiveness of the antisera varies greatly depending upon a large number of factors including, for example, the composition and titer of the specific antibodies, which cannot be easily standardized. The use of these antisera may also carry a risk of transmission of viral infectious diseases.

Patients or potential donors of hyperimmune sera have been vaccinated (i.e. actively immunized) with various immunogens in an attempt to stimulate the host synthesis of cross-reactive anti-endotoxin antibodies. Various vaccine compositions and methods of immunization have been studied over the last two decades. See, e.g., Bhattacharjee A, WO 95/29662; McCabe WR, J Infec Dis 1988; 158:291; Greisman SE, Proc Soc Exp Bio Med 1978; 158:482; Goto M, Res Comm Chem Path Pharm 1992; 76:249; DeMaria A, J Infec Dis 1988; 158:301; Baumgartner JD, J Infec Dis 1991; 163:769; Cross A, J Infec Dis 1994; 170:834; Cryz SJ, US 4,755,381; Miler JM, J Med Microbiol 1977; 10:19; Ashton, FE, Microb Pathog 1989; 6:455; Dorner F, US 4,946,677; Cryz SJ, US 4,771,127; Collins MS, US 4,693,891; Pier GB, US 4,285,936; Cryz SJ, J Infec Dis 1991; 163:1040; Cryz SJ, J Clin Invest 1987; 80:51; PCT WO92/06709; US 5,641,492.

Vaccines should avoid the common side effects -- fever, malaise, and other forms of toxicity -- in the animals and humans receiving them, as reported, for

example, in (Ziegler et al., New Eng. J. Med., 307:1225 (1982); DeMaria, Infec. Dis. 158:301 (1988). These side effects may be exacerbated in high risk individuals, such as pre-surgical elderly and sick patients with multiple medical problems, an important target population for the vaccine.

A study has been published in which the concentration of anti-endotoxin-core antibodies was measured in 301 patients prior to cardiac surgery and the relationship to postoperative outcome tested. Bennett-Guerrero et al. J. Am. Med. Ass. 277:646 (1997). These anti-endotoxin-core antibodies were measured using an ELISA which allegedly detects antibodies to the core of LPS. This study found that patients with a higher level of core-specific antibodies were less likely to die or have a prolonged hospital length of stay associated with complications potentially attributable to endotoxemia. This publication did not describe a method for controlling or treating endotoxemia in these patients.

Regarding the toxicity of LPS, there is some evidence that the toxicity of certain types of lipid A or LPS can be reduced in several ways.

Liposomes have been suggested as carriers for lipid A containing agents. For example, incorporation of LPS into liposomes was shown to reduce the toxicity of LPS from Neisseria meningitidis in an attempt to create a vaccine specific for *N. meningitides* LPS. Petrov et al., Infect. Immunity 60:3897 (1992). Other prior art showed that the incorporation of a chemically altered form of lipid A called monophosphoryl lipid A (MPLA) into liposomes resulted in reduced toxicity of the MPLA component. Richards et al. Vaccine 7:506 (1989). It appeared as if incorporating the MPLA and malaria immunogen into the liposomes resulted in an adjuvant effect, that is, incorporation into liposomes increased

the immunogenicity of the malaria immunogen component of this anti-malaria vaccine.

Methods to detoxify the lipid A component of LPS have also been previously described. Bhattacharjee et al. J. Infec. Dis. 173:1157 (1996).

Genetic alteration of bacterial stains has been reported in which the resulting bacteria contain a LPS whose lipid A component causes less biological toxicity. Somerville et al., J. Clin. Invest. 97:359 (1996).

Summary of the Invention

Vaccination (active immunization) with complete core rough LPS antigen particularly from *E. coli* K12 provides both strain-specific protection and cross-core protection without unacceptable toxicity or other side effects. An antigen is considered a complete-core, rough LPS in that it includes, at a minimum, Lipid A, heptose and 3-deoxy-D-*manno*-2-octulosonate (KDO) residues, as well as the outer core galactose and glucose residues. Typically, it also includes the outer core N-acetyl-D-glucosamine residues. For example, it includes the outer core structure of Rb and typically also the structure of Ra, as shown in Fig. 2. It does not include the O-polysaccharide outer region (also called O-polysaccharide side chain).

Thus, one aspect of the invention generally features a method of reducing the adverse effects of endotoxemia in a warm-blooded animal (a mammal, typically a human patient), by administering an effective amount of a composition comprising complete-core, rough, lipopolysaccharide (LPS) antigen (e.g., an Ra LPS) of a Gram-negative bacterium, particularly *E. coli* K12. Preferably, the immunizing composition is a cocktail of complete-core, rough, lipopolysaccharide (LPS) antigen from other Gram-negative bacterium. Useful rough LPSs

are those from E. coli and Salmonella, particularly from each of the five known chemotypes of E. coli: E. coli R1, E. coli R2, E. coli R3, E. coli R4, and E. coli K12 (Jansson et al., Eur. J. Biochem. 115:571 (1981)). See Fig. 3. Only one core structure accounts for all known Salmonella species, and any Ra Salmonella strain can be used, for example Salmonella minnesota R60. Rietschel et al. Infect. Dis. Clin. N. Am. 5:753 (1991). Complete core LPS lacking polysaccharide side chains from other Gram-negative bacteria that may be useful include those from the family Enterobacteriaceae (i.e. the genera Escherichia, Salmonella, Klebsiella, Citrobacter, Shigella, Proteus, Edwardsiella, Enterobacter, Hafnia, Serratia, Providencia, Morganella, Yersinia, Erwinia), the family Pseudomonadaceae, e.g, Pseudomonas aeruginosa and the family Bactoroides, e.g., B. fragilis. See, generally, ESSENTIALS OF MEDICAL MICROBIOLOGY, 3'rd Ed., Volk, et al., pp. 397 and 416 (J/P. Lippencott Co. Philadelphia, PA (1986) for a compilation of Gram-negative bacteria. The composition may include a complete-core, rough, LPS antigen from several (two, three, four or more) Gram-negative bacteria, each of which is different (e.g., different species or at least different strains of the same species) from the other. In such mixtures, the core antigen from each of the four bacteria may be present in functionally equal amounts (e.g., in amounts which are intended to maximize the expression of the common core epitope(s)).

Desirably, vaccines should cause the patient to produce an antibody that binds to an epitope in the core region of the LPS core of at least one Gram-negative bacterial strain whose LPS is not part of the composition, thereby providing for cross-reactivity and cross-protection. It is difficult to achieve genuine vertical and genuine horizontal cross-reactivity and

cross-protection against smooth and rough gram negative LPS, in particular in *E. coli*, the species most commonly isolated from surgical and intensive care unit patients. Cross-reactivity is of two kinds, which may be described as horizontal and vertical. Vertical cross-reactivity refers to an antibody's reaction with LPS's within the same strain that are different sizes, i.e., having different degress of substitution or length of the O-specific side chain. Horizontal cross-reactivity refers to an antibody's reaction with core structures that are different -- i.e., different strains, species, etc. In particular, the patient's antibody response desirably will bind and protect against smooth as well as rough forms of LPS. Without wishing to bind ourselves to any particular theory, we believe that the epitope of the immunogen used in the vaccine according to the invention is accessible in both smooth and rough forms of LPS.

It may be particularly useful to include the antigen in a liposome structure. For example, the ratio (weight:weight)of lipid in the liposome to the LPS antigen is between 1:1 and 5000:1 (more typically between 10:1 and 1000:1). The liposome may include a component to provide stability or alter the compound's charge, selected from the group consisting of: phospholipid, cholesterol, positively charged compounds, negatively charged compounds, amphipathic compounds. Multilamellar type liposomes (MLV) in particular may be used. Small or large unilamellar liposomes (SUVs and LUVs) also may be used.

The composition may be administered intramuscularly intravenously, subcutaneously, intraperitonealy, via the respiratory tract, or via gastrointestinal tract. The dose of antigen can be readily determined by standard dosage trials which correlate dosage with titre and/or protection. A

functional dosage may be between 0.01 ng and 1000 ng per kilogram of patient body weight, but further optimization may indicate that higher dosages (up to 100 μg/kg of body weight) are desirable consistent with safety and avoiding untoward side effects. IgM antibodies can provide suitable protection, and, were the goal is generation of IgM antibodies, the composition may be administered sufficiently in advance to permit IgM antibodies to be produced (at least 2 days more typically longer) prior to potential endotoxin exposure. Also in that case, the composition would not be administered so far in advance that the IgM response deteriorates substantially -- e.g, less than 14 days prior to exposure. The composition may be administered in multiple doses, the first of which is administered at least 2 days prior to potential endotoxin exposure.

Antigen in the composition may be present as part of bacteria that have been killed e.g., by heat or formaldehyde. Alternatively, the antigen may be separated from the bacterium before formulation of the composition. Alternatively the LPS antigen can be in the form of purified LPS or complexed to an acceptable carrier. Appelmelk et al., J. Immunol. Meth., 82:199 (1985).

The antigen may be chemically detoxified. The bacterium may be genetically engineered for various reasons, e.g., to reduce toxicity. The composition may also include an adjuvant, e.g., alum.

The invention also features vaccine compositions described above in connection with the method. Thus, the vaccine is comprised of an effective amount of one or more complete-core, rough, LPS of a Gram negative bacteria. Upon administration to a warm-blooded animal the compositions stimulate the synthesis of antibodies which recognize an epitope in the core region of the LPS molecule and which are cross-protective against

endotoxemia caused by at least two different Gram-negative bacterial strains having different core structures. In particular, the antibodies synthesized in response to the vaccine are cross-protective against smooth LPS as well as complete core rough LPS (lacking O-polysaccharide side chains). In E. coli, the antibodies induced by the vaccine preferably react with all common smooth strain isolates, and preferably also with rough forms of all five core types (R1, R2, R3, R4, and K12). Preferably the antibodies induced by the vaccine are also reactive with both smooth and rough forms of LPS of different strains of Salmonella.

Furthermore, the vaccine described in this invention preferably causes no unacceptable toxicity following its administration to mammals. Toxicity may be controlled by incorporating the LPS into liposomes, by detoxifying the LPSs lipid A component and/or by alteration of the lipid A component by genetic manipulation of the above mentioned bacterial strains.

The vaccine composition can be used to immunize a donor, from whom antibodies are harvested for administration to a patient. Preferably the antibodies harvested comprise a substantial percentage of IgM class antibody.

Another aspect of the invention features a method of quantitating lipopolysaccharide incorporated into liposomes (PAS method). This method, unlike e.g., typical radiolabelling methods, does not require conversion of the lipopolysaccharide to a form which is unsuitable for clinical use.

Other features and embodiments of the invention will be apparent from the following description of specific embodiments.

Brief Description of the Drawings

Fig. 1 is a diagrammatic representation of smooth and rough LPS.

Fig. 2 is a diagrammatic representation of the chemical structure of Salmonella R-mutants.

Fig. 3 is a diagrammatic representation of the chemical structure of known core types found in Salmonella and Escherichia complete core rough LPS.

Description of the Preferred Embodiments

Medical Indications

The patients to be treated with the vaccine include those at risk for endotoxin exposure. Specific candidates for active immunization include patients scheduled for surgery, patients subjected to chemotherapy or radiation therapy as well as burn patients, trauma patients, dialysis patients and hospitalized (particularly ICU) patients, whether or not they exhibit sepsis or septic shock. Other potential candidates for vaccination include members of the military, fireman, and policeman, as well as endurance athletes and livestock such as horses or cows.

LPS Component

As described above, the LPS antigen to be included in the vaccine can be any complete core LPS lacking O-polysaccharide side chains, preferably from *E. coli* K12, as described below. Although Rb chemotypes may be used, the preferred embodiment is Ra or complete core chemotypes. LPS can be purified from cultured bacteria or purchased commercially, e.g., from Difco, Sigma, List Biologicals, in Campbell, California. The organisms in question are widely available from depositories, including the National Culture Type Collection in England (NCTC); the University of Edinburgh collection in

Edinburgh Scotland,[1] and the Forschungsinstitut in Borstell (FB), Germany D-2061. Examples of specific bacterial include, but are not limited to, the following strains: E. coli K12 -- e.g., Edinburgh #MPRL2320; FB W3100 or List Biologicals; E. coli R1 -- e.g.Edinburgh #MPRL2316 or FB F470; E. coli R2 -- e.g., Edinburgh #MPRL2317 or FB F576; E. coli R3 -- e.g., Edinburgh #MPRL2318 or FB F653; E. coli R4 -- e.g., Edinburgh #MPRL2431 or FB F2513; Ra S. minnesota R60 Edinburgh #MPRL1265 or List Biologicals; S. typhimurium Ra (e.g. TV119,1542), P. aeruginosa PAC611 (e.g, Edinburgh #MPRL1091) and K. aerogenes M10B (e.g, Edinburgh #MPRL0954), S. minnesota Rb chemotype (e.g. Edinburgh #MPRL1091) R345); Bacteroides fragilis -- NCTC 9343; B. vulgatis NCTC 10583; B. thetaiotaomicron NCTC 10582.

Without wishing to bind ourselves to a single theory by which the invention operates, we note that the inner core region may contain an important epitope in terms of stimulating the synthesis of cross-reactive and cross-protective anti-LPS antibodies. However, sufficient outer core structures may be necessary to maintain the inner core epitope in a three-dimensional structure which is similar to that encountered in clinically significant LPS isolates (i.e. smooth and rough forms of complete core LPS). The absence of polysaccharide side chain (i.e. rough LPS) allows the core epitope to be the dominant epitope. In smooth forms of LPS, the polysaccharide side chain is a much more dominant epitope than the core thus significantly reducing the relative amount of anti-core antibody produced. In other words, vaccines containing smooth LPS elicit primarily a serotype specific (i.e. anti-polysaccharide side chain) antibody response as opposed

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to the anti-core response which is the focus of our invention.

E. coli K12 may be particularly useful because it is not generally present in the patient population. Therefore, K12 is less likely to provoke a memory response to the outer core, and more likely to provoke a cross-reactive memory response to the inner core.

If whole bacteria are to be included in the vaccine the bacterium will be killed by a technique well known to those in the art, such as heat killing or formaldehyde killing. In this case, the entire LPS of rough mutant bacterium will be included as part of the killed bacterium. It is desirable to avoid bacterial killing methods which can alter the core.

Alternatively, complete core LPS can be isolated from the desired bacteria according to standard techniques as outlined by Hancock et al., BACTERIAL CELL SURFACE TECHNIQUES, pp. 91 (John Wiley & Sons 1988). As noted, it is preferable to include all of the core LPS, without the O-polysaccharide outer LPS structures, i.e. use R-mutant bacteria expressing full LPS core.

Patient Response

As noted, the desired patient response is cross-protective antibodies that bind to the core of rough and smooth LPS of Gram-negative bacteria generally, regardless of whether their outer LPS structures are similar.

For example, in E. coli, the antibodies induced by this vaccine preferably react with all common smooth strain isolates, and preferably also with rough strain LPSs of all five core types (R1, R2, R3, R4, and K12). Preferably the antibodies induced by this vaccine are also reactive with different smooth and rough LPSs of Salmonella.

It is possible to achieve a vigorous and effective antibody response using compositions with acceptable levels of (or no) toxicity. The vaccine stimulates the synthesis of antibodies which recognize an epitope in the core region of the LPS molecule and which are cross-protective against endotoxemia caused by at least two different Gram-negative bacterial strains having different LPS structures and in particular are cross-protective against smooth strains as well as complete core rough strains.

Typically, the vaccine will be a cocktail of the purified LPS from different strains of bacteria, preferably rough strains having a complete core, for example a mixture of LPS from K12 with LPS from R1 and R3 rough strains of E. coli, or with the Ra strain of Salmonella minnesota R60. E. coli R2 and R4 are less important but also candidates. Preferred cocktails (depending on the breadth of protection desired) include K12 with R1; K12 with Pseudomonas (e.g., P. aeruginosa) and Klebsiella (e.g., K. aerogenes). Since the Bacteroides are a particularly significant population in the gut, it may be important to protect specifically against Bacteroides endotoxin by including Bactoeroides in the cocktail. e.g., together with K12 or together with K12, Pseudomonas and Klebsiella.

Alternatively, the purified LPS from one of these strains, a mixture of any combination of these strains, or a different strain of bacteria may be used in any ratio of the individual strains in the case of use of more than one LPS type.

The route of administration is preferably subcutaneous or intramuscular, although any alternative route which results in these immunogens reaching the antigen presenting cells and antibody producing cells is acceptable. Some other examples include but are not

limited to intravenous, intraperitoneal, and via the respiratory or gastrointestinal tract.

The dose of this composition should stimulate the host to produce increased quantities of cross-reactive and cross-protective antibodies levels, consistent with avoiding toxicity, as described above.

The composition is administered before endotoxin exposure. To the extent that the vaccine works in part by stimulating the host to synthesize antibodies of the IgM class, the vaccine is preferably given between 2 to 14 days prior to potential endotoxin exposure. Alternatively, additional doses of any of the possible permutations of this vaccine may allow for greater effectiveness and increases in desired antibody levels or even further reduced toxicity. It is anticipated that in most vaccinees the antibody response to inner core determinants will be a secondary (i.e. memory) response as opposed to a primary (i.e. naïve) response. This is because most vaccinees will have been exposed at some time in their lifetime to the LPS core epitopes, presumably from LPS that has leaked through the gut barrier into the bloodstream. In other words, an important function of our method of vaccination is to cause an increase in the serum concentration of antibodies which may already be present, but at levels which do not allow for sufficient protection from a toxic exposure of LPS during periods of stress and trauma. The above in no means suggests that there are not patients who will also benefit from vaccination with this invention by means of a primary (i.e. naïve) antibody response.

A vaccine with the LPS mentioned above is preferably rendered non-pyrogenic and non-toxic by incorporation of the LPS into liposomes. The liposome (exclusive of the LPS component) may contain a

combination of (1) a phospholipid and cholesterol or (2) a phospholipid, cholesterol and a negatively or positively charged (lipophilic) amphipathic compound. The phospholipid component may be selected from the group comprising any lipid capable of forming liposomes, including, but not limited to: any phosphatidyl-choline derivative, glycerophosphatides, lysophosphatides, sphingomyelins, and mixtures thereof. The negatively charged (lipophilic) amphipathic compounds may be selected from the group comprising di(alkyl)phosphates, phosphatidic acid, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, dicetyl phosphate, or any other similar negatively charged amphipathic compound that can impart a negative charge to a liposome surface. When positively charged (lipophilic) amphipathic compounds are employed; they are selected from the group comprising alkyl amines, such as stearylamine and hexadecylamine. The ratio in the constituents of the liposomes (exclusive of the LPS) will effect the liposomes' charge, rigidity, stability and may vary greatly while still allowing for reduced toxicity and increased immunogenicity of the LPS they contain. Polyethylene glycol lipids (PEG) may be incorporated into the liposomes, for example at approximately 10 to 20 mole%, in order to increase the amount of time that the liposomes remain in the systemic circulation, thus affecting their immunogenicity. Alternatively, very rigid bilayers may be made by using lipids which are gel phase at body temperature (37 degrees C), for example distearoyl phosphatidylcholine or distearoyl phosphatidylserine. The type of liposomes used is preferably multilamellar liposomes (MLV) but alternatively upon sonication, or by alternative methods of manufacture, small or large unilamellar liposomes (SUVs and LUVs) of varying sizes can be employed.

Different salt forms of LPS may alter the degree of incorporation of LPS into the liposomes, for example, the acid salt form, magnesium salt form, and calcium salt form may allow for increased incorporation due to their increased hydrophobicity.

Liposomes are defined as closed vesicles, or sacs, which contain phospholipids (examples of which are lecithin and sphingomyelin) and which may contain other lipids (examples of which are cholesterol and other steroids; charged lipids such as dicetyl phosphate and octadecylamine; glycolipids; fatty acids and other long-chain alkyl compounds; hydrophobic glycoproteins; and lipid soluble vitamins and lipoidal surfactant-like molecules). When shaken in the presence of an excess amount of water, the lipid mixture is formed into discrete particles consisting of concentric spherical shells of lipid bilayer membranes which are referred to as multilamellar liposomes (MLV). Upon sonication, or by alternative methods of manufacture, small or large unilamellar liposomes (SUV or LUV, respectively) can be formed.

Upon injection into animals and man, liposomes are taken up rapidly by cells of the reticuloendothelial system, particularly those of the liver. Because of the relative impermeability of liposomes and their speedy removal from the circulatory system, substances such as lipid A and certain forms of LPS remain incorporated within the liposomes and are less likely to be exposed to cells and/or receptors through which they can exert potentially toxic effects. Moreover, liposomes may allow for a prolonged effectiveness through slow biodegradation of the multilamellar membrane structure of the liposomes.

The toxicity of the lipid A component of the above mentioned complete core rough mutant strains also can be reduced or eliminated by chemical detoxification as

described in Bhattacharjee A et al., WO 95/29662. The preferred method for this detoxification maintains the LPS configuration such that it still stimulates the synthesis of antibody/ies which recognize an epitope in the core region of the LPS molecule and which is cross-protective against endotoxemia caused by at least two different Gram-negative bacterial strains having different core structures. In particular, the antibodies synthesized in response to this vaccine are cross-protective against smooth strains as well as complete core strains. The detoxified LPS may be administered in the form of purified LPS, or alternatively can be incorporated into liposomes or complexed to an acceptable carrier.

Alternatively the toxicity of the lipid A component of the above mentioned strains of bacteria can be reduced or eliminated by genetic alteration of the bacterial strains as described in Somerville JE et al, J Clin Invest 1996; 97:359-365. The resulting LPS from these cells (in the form of heat killed cells) is reduced in toxicity while still affording immunogenicity to LPS core. The preferred method for this genetic alteration maintains the LPS in a sufficient three-dimensional shape that it still acts sufficiently as an immunogen in a host to stimulate the synthesis of antibody/ies which recognize an epitope in the core region of the LPS molecule and which is cross-protective against endotoxemia caused by at least two different Gram-negative bacterial strains having different core structures. In particular, the antibodies synthesized in response to this vaccine are cross-protective against smooth strains as well as complete core rough strains. At the same time, this genetic process preferably renders the LPS non-pyrogenic and non-toxic in the warm-blooded animal. The LPS from these genetically altered bacterial

strains are preferably administered in form of purified LPS incorporated into liposomes. LPS from these altered strains can alternatively be administered in the form of killed cells. Alternatively, the detoxified LPS may be administered in the form of purified LPS, or alternatively can be complexed to an acceptable carrier.

Toxicity of any of the LPS rough antigen compositions described in this invention may also be reduced by other methods, for example, competitive detoxification of lipid A by synthetic anti-endotoxin peptides. Rustici et al. Science 259:361 (1993). An alternative method of reducing toxicity is to administer the LPS antigen with or at around the same time as an anti-inflammatory agent, e.g., anti-TNF-alpha monoclonal antibody. Fisher CJ et al. N Engl J Med 334:1697 (1996).

EXAMPLES

Examples herein offered to illustrate the invention are not intended to limit the scope thereof. These examples are offered to indicate experiments that may be done, with no implication that all or any of the experiments have in fact been performed.

Materials for liposome preparation

Synthetic dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl serine, and dimyristoyl phosphatidyl glycerol (DMPG) were purchased from Avanti Polar Lipids. Cholesterol, 3-(N- Morpholino) propane sulfonic acid (MOPS), periodic acid, and pararosaniline based Schiff's reagent are purchased from Sigma. Sterile saline and water for irrigation are purchased from Abbott labs. 3M Empore solid phase extraction discs are obtained from Fisher. Limulus amebocyte lysate (LAL)

standards and reagents are obtained from Associates of Cape Cod.

Materials and reagents are depyrogenated in one or more of the following procedures: 1) Heating- glassware are heated to approximately 180 degrees for no less than 16 hr; 2) Base Treatment- immersed in isopropanol/concentrated potassium hydroxide for not less than 2 hours; 3) Hydrogen Peroxide- immersed in concentrated hydrogen peroxide for not less than 1 hour at 70 degrees Celsius then rinsed with depyrogenated water; 4) Ultrafiltration- solutions are filtered through Amicon centriprep ultrafiltration membranes (3000 M.W.).

All glassware, plasticware, solutions, and buffers are free of contaminating endotoxin and verified by use of the standard LAL assay (Pyrotell and Pyrochrome from Associates of Cape Cod, Cape Cod, MA, USA).

MOPS Saline Buffer and 0.5% periodic acid solutions are stored at room temperature prior to use. Lipid stock solutions are stored at -20 degrees Celsius. LPS is stored dissolved in water for irrigation or in 0.1% TEA in glass or polystyrene containers (Evergreen) at 4 degrees Celsius. Liposome preparations are stored at 4 degrees Celsius. Pararosaniline based Schiff's reagent is stored at 4 degrees Celsius prior to use.

Methods

Lipopolysaccharide

Established strains of the following bacteria are maintained according to standard procedures: K12, R1, R2, R3, and R4 rough strains of E. coli, E. coli smooth strains O18, 06, 0157, 012, 015, and the Ra strain of Salmonella minnesota R60 can be obtained as described above. Smooth and rough LPS are purified according to the established method described in Hancock et al., cited above, pp 91-92. LPS from E. coli J5 (Rc chemotype), E. coli, S. minnesota R595 (Re chemotype), and S. minnesota

(wild type), and S. typhimurium (wild type) can be obtained from List Biological Laboratories Inc. (Campbell, Calif). Native LPS may be used as well as acid form (deionized) LPS made by electrodialyzing the native LPS using the established method described in Hancock et al. cited above at 93-95.

Incorporation of LPS into Liposomes

Liposomes are prepared by standard procedures. Multilamellar vesicles (MLV) are prepared according to the method of Dijkstra et al, J Immunol Methods 1988; 114:197-205, with some modifications, as indicated in Methods #1 and #3, below, or a novel method (method #2).

For example, in method #1, 1 ml of 5 mg/ml of native *E. coli* K12 (Ra) LPS in water was added to a 1 ml aqueous dispersion of 50 mg lipid (DMPC:DMPG:cholesterol, 4:1:4, mol/mol), i.e. a lipid:LPS ratio of 10:1 (wt/wt). The combined solution was then probe sonicated at 40-50 degrees Celsius for five 2 minute periods with 2 minute wait periods in between each sonication. The solution was then rotovapped to dryness and resuspended in a buffer consisting of 4 mM MOPS, 153 mM NaCL, ph 7.8. LPS not incorporated into the liposomes (free LPS) was removed by centrifuging the preparation 3 times at 10,000 rpm in a Komp Spin KA 21.5 rotor for 10 minutes, decanting all supernatant, and resuspending the pellet in the original volume of buffer. This procedure was repeated 3 times and reduced the concentration of unincorporated LPS significantly. This method for reducing free LPS can be used following most liposome preparation methods.

In method #2, a solution of 5 ml chloroform:methanol (2:1, v/v) was vortexed together with 2 ml of 0.1 M HCl. The lower organic phase was then allowed to separate from the upper, removed, and used to

dissolve 5 mg of acid form LPS. 50 mg of lipid (DMPC:DMPG:cholesterol, 4:1:4, mol/mol) was then disolved in the LPS solution. The solution of codisolved LPS and lipid was rotovapped to dryness, and resuspended as in method #1.

In method #3, briefly, a 1 ml of a lipid stock solution consisting of 4 mM dimyristoyl phosphatidyl choline:1mM dimyristoyl phosphatidyl serine: 4 mM cholesterol was rotovapped to dryness at 50 degrees Celsius. 50 μl of a 0.1 mg/ml solution of mixed LPS consisting of equal weights of LPS from Escherichia coli strains K12, R1, R2, R3, R4 and Salmonella minnesota R60 suspended in 0.1% TEA is added with 150 μl of water. The preparation was then vigorously vortexed and sonicated for not less than 5 minutes in a bath type sonicator with hot tap water in the bath. The preparations were then rotovapped (or lyophilized) to dryness again and resuspended in a buffer consisting of 4 mM MOPS 153 mM saline pH 7.8 with vigorous vortexing.

In some of the methods of incorporation of LPS into liposomes a single LPS type was used (e.g. 5 mg of E. coli K12 (Ra)) while in others, equal amounts of different LPSs were used (e.g. 0.83 mg of LPS from 6 complete core rough mutants).

Large unilamellar vesicles (LUV) were prepared from MLV by repeated (minimum of 15) passages through a depyrogenated pair of 100 nm polycarbonate membranes housed in an Avestin Liposofast extruder which was also depyrogenated prior to use by the hydrogen peroxide treatment above.

LPS incorporated into liposomes from method #3 was purified on a 1.1 x 28.5 cm BioGel A15M column using 4 mM MOPS/153 mM saline as the running buffer and 0.7 ml/min as the flow rate. This step maybe unnecessary since there was no difference in the lymulate ameoba lysate

(LAL) activity between purified and unpurified liposomes due to the almost complete (>99.9%) incorporation of the free LPS into the liposomes.

In vitro Quantitation of Toxicity of LPS

Quantitation of the biological activity of the toxic lipid A component of all samples of vaccine and controls was accomplished using the standard LAL assay according to the manufacturer's instructions. (Pyrotell and Pyrochrome from Associates of Cape Cod, Cape Cod, MA, USA). The rate of incorporation of free LPS into liposomes is generally reflected by a significant decrease of LAL activity following effective incorporation. Rates of incorporation of LPS into liposomes using traditional methods, including those described above, usually exceed 90% and in some of the methods exceeded 99%.

Periodic Acid/Schiff's Base (PAS) Stain for LPS

Aliquots of LPS containing either MLV or LUV are diluted to 2 ml with water and extracted with 2 ml toluene. After vortexing and a 5 minute 2800 rpm centrifugation in a Sorvall GLC-2 centrifuge, the upper toluene phase is removed and the aqueous phase is reextracted with 2 ml of fresh toluene. The aqueous phase and interphase are then taken, acidified with 30-50 μl of concentrated hydrochloric acid and extracted with 5 ml of chloroform:methanol (2:1). The aqueous phase is reextracted once with chloroform and the combined organic phases are dried under nitrogen at 50-60 degrees Celsius. Residues are then dissolved in chloroform:methanol (2:1) and spotted on an Empore C8 extraction disc alongside an LPS standard curve spotted from 50% ethanol. Discs are dried in vacuo and incubated in 0.5% periodic acid at room temperature for approximately 30 minutes. They are then removed, rinsed with distilled water, and placed in

capped test tubes containing pararosaniline based Schiff's reagent. The tubes are then warmed under hot tap water until color develops. The discs are then removed, rinsed again, and dried. Quantitation of the resulting spots is done on an Agfa Arcus II desktop scanner in conjunction with Adobe Photoshop and NIH Image software.

Immunization and Pyrogenicity testing

The pyrogenicity and toxicity of a vaccine comprised of LPS is best measured in the rabbit model of pyrogenicity established and outlined in the United States Pharmacopiae. (USP 23, <151>, 1995, Rockville, MD) In this established protocol, if an experimental substance is administered and does not cause pyrogenicity in rabbits relative to control animals, that substance is defined as being non-pyrogenic and is unlikely to cause fever or toxicity in other mammals, particularly humans, following its administration. This protocol is an ideal model since rabbits and humans are similarly sensitive to endotoxin.

Briefly, mature female New Zealand White rabbits between 1.8 and 3.0 kg were sham tested twice to insure their suitability for pyrogenicity testing. All testing materials were administered as specified, either intravenously (IV) as a 1 ml volume or intramuscularly (IM) as a 0.6ml volume. Three rabbits are used per group as necessary for tests completed to comply with regulatory requirements. However, 2 rabbits per group were sufficient to demonstrate large differences in pyrogenicity between free LPS and LPS incorporated within liposomes in examples described below. Temperatures of the rabbits were measured to insure a steady baseline and following administration of the test sample were monitored at 15 minute intervals for 3 hours. A material

is considered non-pyrogenic if no rabbit shows an individual rise in temperature of >0.5 degrees C above baseline.

Measures of Efficacy of Various Immunogens

A well recognized test for the effectiveness of an immunogen is to administer the immunogen to a warm blooded animal, typically a rabbit or a human subject, and then withdraw blood samples periodically for the determination of antibody levels. Blood samples were drawn from a marginal ear vein into a red top tube the day before testing and at different time points following intramuscular (thigh muscle) immunization. Some intramuscular immunizations were performed with the administration of the adjuvant Alum. In these cases 0.3 ml (equal to 1.50 mg) of Alum (Alhydrogel 0.5% diluted from stock 2%, Sergeant Co., Clifton, NJ) was mixed thoroughly with 0.3 ml of vaccine immunogen prior to administration. Blood was centrifuged, the serum removed, and stored at -80 degrees C.

Binding of immunized rabbit serum to LPS by ELISA

The cross-reactivity of sera from the immunized rabbits was determined by a standard method of binding of sera to purified LPS in enzyme-linked immunosorbent assay (ELISA). Sera was obtained from rabbits before and after immunization with different possible immunogens. This sera was tested against numerous purified LPS in order to determine the degree of horizontal cross-reactivity and vertical cross-reactivity of the sera. The rabbit serum following the method of active immunization described in this invention bound to smooth and complete core rough LPS of Escherichia coli as well as smooth and complete core rough LPS of Salmonella. It demonstrated superior binding than sera from rabbits immunized with immunogens

from prior art such as the extensively tested Rc J5 mutant of Escherichia coli, the Re mutant of Salmonella minnesota, and lipid A.

LPS-polymyxin complexes

LPS molecular weights were calculated as described previously (Scott BB et al., Serodiag Immunother Infect Dis; 4:25(1990)). Purified LPS at 0.2mM in pyrogen-free water (5ml) were mixed with polymyxin B sulphate (Sigma) at 0.4mM in pyrogen free water (5ml), and sonicated together with approximately ten short (5 second) bursts of sonication. The resulting milky suspension was placed in a 2000 MWCO membrane and dialysed overnight against freshly-distilled water containing 0.05% Na axide (w/v) to remove excess uncomplexed polymyxin B. The dialysed material, often presenting as a flocular precipitate, was recovered as a 10ml suspension with a presumptive LPS concentration of 0.1 mM, and stored in polypropylene Minisorb tubes (Nunc) at -40C.

LPS coating on microplates.

LPS-polymyxin complexes were resuspended with sonication. LPS-polymyxin complexes were diluted 1:80 in 0.05 M carbonate-bicarbonate buffer pH 9.6, containing 0.05% sodium azide, which had been freshly prepared using freshly-distilled water. The diluted complexes (containing 1.25 micromolar LPS) were maintained in even distribution in coating buffer by continuous rapid stirring, and added at 100 microlitres per well to 96-well microtitre plates or 8-well microtitre strips (in 96-well frames). The microtitre plates and strips used were ELISA-grade polystyrene (Greiner, medium-binding grade, flat-bottom wells): some other grades and some other manufacturers microtitre plates may be unsuitable for this assay. Plates were stacked, wrapped in plastic (Clingfilm), and incubated overnight at 37C. The plates were washed as previously described using phosphate-

buffered saline (PBS) with 0.05% (v/v) tween-20. A 5% solution of bovine serum albumin (BSA) in PBS, containing 0.05% sodium azide, was added at 120 microlitres per well. The plates were stacked, wrapped in plastic, and incubated overnight at 37C. The plates were washed as before, rinsed using freshly-distilled water, blotted by inverting on absorbent paper, and dried at 37C. The dried plates were sealed in plastic bags (one plate per bag) and stored at -40C until used.

LPS ELISA

Samples of test serum or plasma were diluted 1:200 in ELISA diluent [PBS / tween-20 (0.05% v/v) / polyethylene-glycol 8000 (4% w/v) / BSA (1.0% w/v) / sodium azide (0.05% w/v)], and added at 100 microlitres per well, in triplicate, to LPS-coated plates. Plates were incubated at 37C for 5 hours in a still-air (no fan) incubator, then washed (PBS / tween). Other dilutions, incubation durations, and test replication may be used, however, for any given set of experimental control and experimental groups should be subjected to identical conditions. An alkaline-phosphatase-conjugated species-specific, immunoglobulin heavy-chain-specific antibody was used to determine the amount of each immunoglobulin class bound. IgM antibodies were determined with mu-chain specific conjugates and IgG antibodies were determined with gamma-chain specific conjugates. Heavy-chain specific species specific antibodies (e.g. anti-rabbit Ig antibodies purchased from Harlan Sera-Lab (UK), were used at 1:1000 in ELISA dilution buffer. The diluted conjugates were added at 100 microlitres per well, and plates were incubated for 120 minutes at 37C. The plates were washed in PBS / tween, rinsed in distilled water, blotted, and 100 microlitres of freshly-prepared pNPP alkaline phosphatase substrate solution (Sigma N-2770) was added per well. The color was allowed

to develop for 30 minutes at room temperature, and plates were read (at 405nm and reference at 650nm) on an automated ELISA plate reader (Molecular Devices Thermo-max) and tests were expressed as the net optical density at 405 nm and 650nm (reference). Alternatively, test results can be read as above and expressed as a percentage of a standard on the same plate using automated curve fitting from device-related software (Molecular Devices Softmax). Test samples can be compared to a laboratory standard imuune serum from the same species, placed as a triplicate series of 8 doubling dilutions in ELISA diluent (standard curve) from, say, 1:50, down one column of triplicate wells on each microplate.

Binding of immunized rabbit serum to LPS by Western Blotting

The cross-reactivity of sera from the immunized rabbits was also determined by the standard method of binding of sera to purified LPS in Western blotting. Sera was obtained from rabbits before and after immunization with different possible immunogens. This sera was tested against numerous purified LPS in order to determine the degree of horizontal cross-reactivity and vertical cross-reactivity of the sera. The rabbit serum following active immunization with the method of active immunization described in this invention bound to smooth and complete core rough LPS of Escherichia coli as well as smooth and complete core rough LPS of Salmonella. It demonstrated superior binding than sera from rabbits immunized with immunogens from prior art such as the extensively tested Rc J5 mutant of Escherichia coli and is expected to demonstrate superior binding compared with serum from rabbits immunized with purified lipid A as well as rabbits immunized with LPS from the Re mutant of Salmonella minnesota.

PAGE analysis was performed on 12% (w/v) acylamide gels with the buffer system of Laemmli (Nature; 227:680-85 (1990)),except SDS was omitted from the stacking and separating gel buffers. Samples of LPS (5-10 micrograms for rough LPS and 20-25 micrograms for smooth LPS, mixed with Laemmli's sample buffer) were loaded onto the gel and electrophoresed at 60 volts until the sample had entered the separating gel, and then at 150 volts until the dye front had migrated 7.5 cm through the gel. The separated LPS was stained by the modified silver stain of Hancock and Poxton (Bacterial Cell Surface Techniques, pub. Wiley, p. 281 (1988)) except that oxidation was done for 15 minutes. For immunoblotting, the LPS was transferred to nitrocellulose membrane (Schleicher and Schuell, Germany), 0.2 micrometer pore size at 10-12 volts for 16 hours at 4 degrees Celsius with the Tris, glycine, methanol buffer of Towbin et al, Proc Natl Acad Sci USA; 76:4350-54 (1979)). The transferred LPS was immunostained as described in Hancock and Poxton (Bacterial Cell Surface Techniques, pub. Wiley, p. 204-5 (1988)), except that incubation times and serum dilutions were selected to give best results, and the immunoblot was rinsed prior to developing.

The LPS content extracted from a smooth bacterium was separated by electrophoresis into bands corresponding to LPS molecules having different molecular weights, depending on the size of the O-specific side chain. These LPS molecules ranged from LPS molecules without any O-specific side chain (equivalent to the size of a complete core (Ra) rough mutant) to LPS molecules having 40 or more units in the side chain.

<u>Protection Based on Inhibition of LPS Induced Stimulation of the LAL Assay by Serum from Vaccinated Rabbits</u>

The limulus amebocyte lysate (LAL) assay is an established test for the biologic activity/toxicity of

lipid A (the toxic component of LPS). This assay quantitates the activity of a biologically active LPS, whereas in the presence of protective anti-LPS antibodies there is significant inhibition of the LAL test due to competitive binding and neutralization of the LPS. There are numerous methods for using the LAL assay to demonstrate protection from anti-LPS antibodies. LAL test kits can be purchased from manufacturers, e.g. Coatest Endotoxin from Chromogenix, Sweden. Different versions of the LAL assay can be used, e.g. gel-clot version or chromogenic version.

In an example of this method, a kinetic chromogenic LAL assay was used. In this assay, serum was obtained from a rabbit before and after vaccination with complete core LPS incorporated into liposomes. E.coli R1 LPS (1000 micrograms/ml) was diluted 1/5 into pyrogen-free water on a microplate, then five-fold diluted across the plate, allowing for a final volume of 40 ul in 5 wells (1000 ug/ml to 1.6 ug/ml). Dilutions of LPS are performed to avoid: 1) having only wells in which there is not enough LPS to cause LAL activation in the presence of serum; 2) having only wells in which there is an excessive amount of LPS. There was a dilution series for each serum or control to be tested (total 3 rows).

Row A- pyrogen-free water
row B- day-0 rabbit serum (complete core immunogen)
Row C- day-63 rabbit serum (complete core immunogen)
20 ul of water or rabbit serum was added to each well and left for 30 minutes at room temperature. 20 ul of LAL/substrate was then added using a multi-tip pipettor (start of reaction) to each well, placed immediately in reader, and read at 20sec intervals for 120 minutes. The time (seconds) from start of reaction to an optical density (OD) of 0.5 is a reflection of the degree/speed of LAL activation. In the presence of water and LPS, the

LAL reaction proceeded quickly and an OD of 0.5 was reached quickly. Pre-immunization serum contains some anti-LPS antibodies as well as non-specific inhibitors of LPS (e.g. lipoproteins). This serum partially neutralized the LPS thus slowing down the LAL reaction and increasing the time required for the OD to reach 0.5. Post-immunization serum from the complete-core immunized rabbit resulted in a significant neutralization/protection of the R1 LPS as evidenced by the marked prolongation of the time required to reach an OD of 0.5. Similar results were obtained using other types of stimulating LPSs and were consistent with the ELISA LPS binding data described earlier.

In another method using inhibition of LAL activity, a known quantity of LPS is added to several dilutions of serum from both pre- and post-vaccinated subjects and the LAL activity (EU) for each is compared. Protective anti-LPS antibodies in serum, in particular the post-vaccination serum, result in lower LAL activity compared with the pre-immunization value.

Cross-Protection Based on Inhibition of LPS-Induced IL-6 Secretion by Murine Peritoneal Macrophages

Several monokines including tumor necrosis factor (TNF), IL-1, and IL-6 mediate many of the pathophysiologic events associated with Gram-negative endotoxemia. These monokines are secreted by monocytes and macrophages both in vitro and in vivo, in response to LPS. Serum with protective anti-LPS antibodies block the LPS induced macrophage or monocyte stimulation as shown in the following assay. This type of assay can be done using established mouse cell lines (e.g. J774.2), established human cell lines (e.g. THP-1), freshly obtained mouse peritoneal cells (C3H/HeN), or freshly obtained human monocytes/macrophages. Following stimulation with LPS, the assay can test for TNF or IL-6

levels. The amount of any type of LPS (e.g. E. coli R1) used to stimulate the cells needs to be determined in preliminary experiments. Too little LPS results in inadequate stimulation and undetectable monokine levels in the control group whereas too much LPS can overwhelm even a large amount of protective antibody. Serum with protective antibodies results in a lower monokine level (for example, the TNF level), compared with serum controls following stimulation with LPS.

In one example, this assay was performed as in Delahooke DM et al. Infection and Immunity, 1995, p 840-46. This assay uses a human cell line (THP1) which secretes TNF following stimulation with LPS. In this assay, the serum after vaccination with complete core antigen demonstrated significant inhibition of TNF induction

In another example, mouse peritoneal cells (C3H/HeN) are obtained by peritoneal lavage with 0.34 M sucrose in distilled water. Peritoneal cells are seeded at $5x10^5$ cells/ml in 0.2 ml serum free medium (IMDM-ATL, Schreier and Tees, Immunological Methods, Vol. II, Acad. Press (1981) : 263) and cultured for 4 hours at 37 degrees C in the presence or absence of (1) LPS, e.g. LPS from E. coli R3 (0.05 ng/ml) or E. coli O18 (0.05 ng/ml) or S. minnesota wild type smooth (0.05 ng/ml) or S. minnesota R60 (0.05 ng/ml); and (2) in the presence or absence of diluted or undiluted serum from rabbits immunized with varying immunogens, e.g. composition described in this invention or Rc J5 mutant of E. coli or Re mutant of S. minnesota. The supernatants are recovered and the amount of IL-6 present in the supernatants is then measured using the IL-6 dependent hybridoma cell-line B13.29 (Aarden et al., Eur. J. Immunol. 1987, 17, 1911) as follows:

B13.29 cells are seeded at 2.5 x 10^4 cells/ml in serum free medium and cultured for 72 hrs in the absence of IL-6 and in the presence or absence of culture supernatant. Aliquots of the cultures (200 μl/well) are distributed in flat bottomed microtiter plates. IL-6 concentration in the supernatants is calculated in relation to a standard curve of IL-6. The post-immune serum should cause reduced IL-6 secretion compared to the pre-immune serum.

Cross-Protection from Lethal Dose of Endotoxin

Another measure of the effectiveness of this invention is its ability to confer cross-protection against LPS. A mouse lethality model is used in which mice are immunized intraperitonealy on Day 0 with either the invention, appropriate controls, or immunogens described previously such as the Rc J5 mutant LPS of Escherichia coli and the Re mutant LPS of Salmonella minnesota. A second dose of antigen is administered on Day 7 and Day 14. Between days 19 and 21 endotoxin can be administered intravenously in a 95% lethal dose of a particular LPS to groups of six female C57BL/6 mice, 6-8 weeks old. Galactosamine (D-GalN) (800 mg/kg) is administered intraperitoneally at the time of the LPS. The minimum intravenous dose of LPS required to kill approximately 95% of the animals (LD_{95}) is determined in preliminary experiments. Survival is recorded up to 24 hours. Alternative methods can be used in this protocol without substantially changing its ability to demonstrate whether immunization with a particular immunogen results in protective antibodies. For example, other strains of mice can be suitable, the galactosamine dose can be modified, and the dosing schedule of vaccination can be altered so as to administer more doses. The experiment can also be performed by isolating

serum from rabbits immunized with the experimental immunogen, and then administering the serum to mice prior to challenge with galactosamine and LPS. This method using passive immunization can be used to demonstrate the protective nature of serum.

Example 1

Liposomes containing *E. coli* K12 complete-core LPS were made according to method #2, above. Liposomes containing a cocktail of six complete-core LPS (*E. coli* R1-R4, K12, S. minnesota R60 Ra) were made according to the method described above.

Groups of three mature rabbits were immunized intramuscularly using a dose of 0.5mg of antigen with Alum (as described earlier) on days 0, 14, and 56. On days 0, 14, 21, 56, and 63, blood was withdrawn and processed as described earlier.

Using the ELISA method described earlier, rabbits immunized with K12 demonstrated increases in both IgM and IgG antibody levels to smooth and rough forms of LPS from E. coli and Salmonella bacteria.

Using the Western/immunoblot method described earlier, serum from both groups of immunized rabbits demonstrated enhanced binding to smooth and rough forms of LPS from E. coli and Salmonella typhimurium. Binding of serum from rabbits immunized to K12 alone to smooth forms of LPS from E. coli (serotypes 018, 012 and 015) as well as to the LPS from Salmonella typhimurium wild type was comparable to binding of serum from rabbits immunized with the cocktail of six complete LPS cores.

Example 2

Purified lipopolysaccharide in equivalent amounts from the following rough strains of bacteria having a complete core, R1, R2, R3, R4, and K12 strains of Ra E. coli and the Ra strain of Salmonella minnesota R60, are tested either alone or following their incorporation into

liposomes as described earlier. Heat killed cells and liposomes alone are also evaluated. Compositions are made according to the materials and methods described earlier.

Groups

1. 100 μg of MLV (no LPS)
2. Purified LPS (3 ng total) from E. coli R1
3. purified LPS (0.3 ng total) (0.05 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60)
4. purified LPS (3 ng total) (0.5 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60)
5. purified LPS (30 ng total) (5 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60
6. purified LPS (300 ng total) (50 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60)
7. Cocktail of the 6 LPSs mentioned above incorporated into MLVs as 1:1000 ratio by weight (LPS:lipid) (3 ng total) (0.5 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60)
8. Cocktail of the 6 LPSs mentioned above incorporated into MLVs as 1:1000 ratio by weight (LPS:lipid) (30 ng total) (5 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60)
9. Cocktail of the 6 LPSs mentioned above incorporated into MLVs as 1:1000 ratio by weight (LPS:lipid) (300 ng total) (50 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60)
10. Cocktail of the 6 LPSs mentioned above incorporated into LUVs as 1:1000 ratio by weight (LPS:lipid) (3 ng total) (0.5 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60)
11. Cocktail of the 6 LPSs mentioned above incorporated into LUVs as 1:1000 ratio by weight

(LPS:lipid) (30 ng total) (5 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60)

12. Cocktail of the 6 LPSs mentioned above incorporated into LUVs as 1:1000 ratio by weight (LPS:lipid) (300 ng total) (50 ng each of E. coli K12,R1,R2,R3, and R4, and S. minnesota R60)

13. Heat killed cells from E. coli R1 (amount that has same LAL activity as 3 ng of E. coli R1 LPS)

What is claimed is:

Claims

1. A method of reducing adverse effects of endotoxin in a warm-blooded animal, which method comprises administering to the warm-blooded animal an effective amount of a composition comprising rough, complete-core lipopolysaccharide (LPS) antigen of *E. coli* K12.

2. The method of claim 1 in which the composition further comprises rough, complete-core lipopolysaccharide (LPS) antigen of a second bacteria other than *E. coli* K12.

3. The method of claim 1 in which the animal is a mammal.

4. The method of claim 2 in which the mammal is a human patient.

5. The method of claim 1 in which the composition comprises LPS of an R_a rough *E. coli* K12.

6. The method of claim 2 in which the second bacterium is an *E. coli* or a *Salmonella* bacterium.

7. The method of claim 2 in which the second bacteria is a Bacteroides.

8. The method of claim 2 in which the composition comprises complete-core, rough, LPS antigen from a third Gram-negative bacterium different from the first and from the second Gram-negative bacterium.

9. The method of claim 8 in which the composition comprises complete-core, rough, LPS antigen from a fourth Gram-negative bacterium different from each of the first, the second, and the third Gram-negative bacteria.

10. The method of claim 2 in which the second Gram-negative bacterium is *E. coli* R1.

11. The method of claim 2 in which the second Gram-negative bacterium is a *Salmonella* bacterium.

12. The method of claim 8 in which the second bacterium is a Klebsiella and third bacterium is a Pseudomonad.

13. The method of claim 9 in which the second bacterium is a Klebsiella, the third bacterium is a Pseudomonad, and the fourth bacterium is a Bacteroides.

14. The method of claim 6 or claim 11 in which the *Salmonella* bacterium is *Salmonella minnesota* R60.

15. The method of claim 9 in which core antigen from each of the four bacteria is present in generally equal amounts by weight.

16. The method of claim 7 in which the composition comprises LPS antigens from at least two different Gram-negative bacterial strains of the same species.

17. The method of claim 1 in which the antigen causes the patient to produce an antibody that binds to an epitope in the core region of the LPS of at least one Gram-negative bacterial strain whose LPS is not part of the composition.

18. The method of claim 17 in which the patient's antibody binds to the LPS of at least one smooth Gram negative bacterial strain.

19. The method of claim 1 in which the composition comprises the antigen in a liposome.

20. The method of claim 19 in which the ratio (weight:weight) of lipid in the liposome to the LPS antigen is between 1:1 and 5000:1.

21. The method of claim 20 in which the ratio (weight:weight) is between 10:1 and 1000:1.

22. The method of claim 19 in which the liposome comprises a component selected from the group consisting of: phospholipid, cholesterol, positively charged compounds, negatively charged compounds, amphipathic compounds.

23. The method of claim 19 in which the liposome is a multilamellar type liposome (MLV).

24. The method of claim 19 in which LPS in the acid salt form is incorporated into the liposome.

25. The method of claim 19 in which the liposome is a small or large unilamellar liposome (SUVs and LUVs).

26. The method of claim 1 in which the composition is administered intramuscularly, intravenously, subcutaneously, intraperitonealy, via the respiratory tract, or via gastrointestinal tract.

27. The method of claim 1 in which the dose of antigen is over 0.01 ng per kilogram of patient body weight.

28. The method of claim 27 in which the dose is less than 1000ng per kilogram of patient body weight.

29. The method of claim 27 in which the dose is less than 100 micrograms per kilogram of patient body weight.

30. The method of claim 1 in which the composition is administered in multiple doses, the first of which is administered at least 2 days prior to potential endotoxin exposure.

31. The method of claim 1 in which the antigen is present in a killed bacterium.

32. The method of claim 1 in which the antigen is separated from the bacterium.

33. The method of claim 1 in which the antigen is chemically detoxified.

34. The method of claim 1 or claim 31 in which the bacterium is genetically engineered.

35. The method of claim 1 in which the composition further comprises an adjuvant.

36. The method of claim 33 in which the adjuvant is alum.

37. A vaccine composition for reducing the adverse effects of endotoxemia in a human patient which comprises an effective amount of a composition comprising purified complete core rough lipopolysaccharide antigen of *E. coli* K12, said composition further comprising liposomes which contain the antigen.

38. A method of reducing adverse effects of endotoxin in a warm-blooded animal, which method comprises administering to the warm-blooded animal an effective amount of a composition comprising rough lipopolysaccharide (LPS) antigen of a Gram-negative bacterium, said LPS antigen comprising the component of an E. coli Rb LPS, or the equivalent thereof in another species.

39. A method of quantitating lipopolysaccharide incorporated into liposomes by performing periodic acid/Schiff base staining.

40. The method of claim 39 in which the test is performed on a vaccine lot intended for clinical use.

41. A method of reducing adverse effects of endotoxin in a warm-blooded animal, which method comprises administering to the warm-blooded animal an effective amount of antibody produce by immunization with a composition accoring to claim 1.

42. The method of claim 41 in which the antibody comprises a substantial percentage of IgM antibody.

43. A method of reducing adverse effects of endotoxin in a warm-blooded animal, which method comprises administering to the warm-blooded animal an effective amount of a composition comprising rough, complete-core lipopolysaccharide (LPS) antigen of a gram negative bacterium.

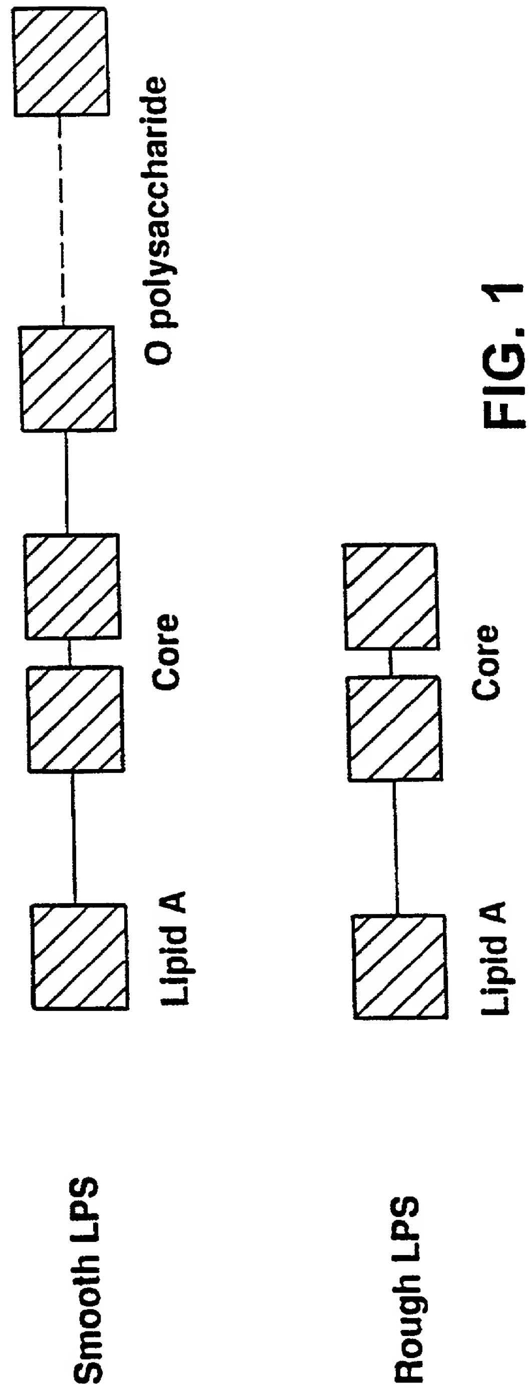

FIG. 1

		Inner core			Outer core		
Ra	Lipid A	Kdo	Hep	Hep	Glc	Gal	Glc
		Kdo		Hep	Gal		GlcNAc
		Kdo					
Rb	Lipid A	Kdo	Hep	Hep	Glc	Gal	Glc
		Kdo		Hep	Gal		
		Kdo					
Rc	Lipid A	Kdo	Hep	Hep	Glc		
		Kdo		Hep			
		Kdo					
Rd	Lipid A	Kdo	Hep	Hep			
		Kdo					
		Kdo					
Re	Lipid A	Kdo					
		Kdo					

FIG. 2

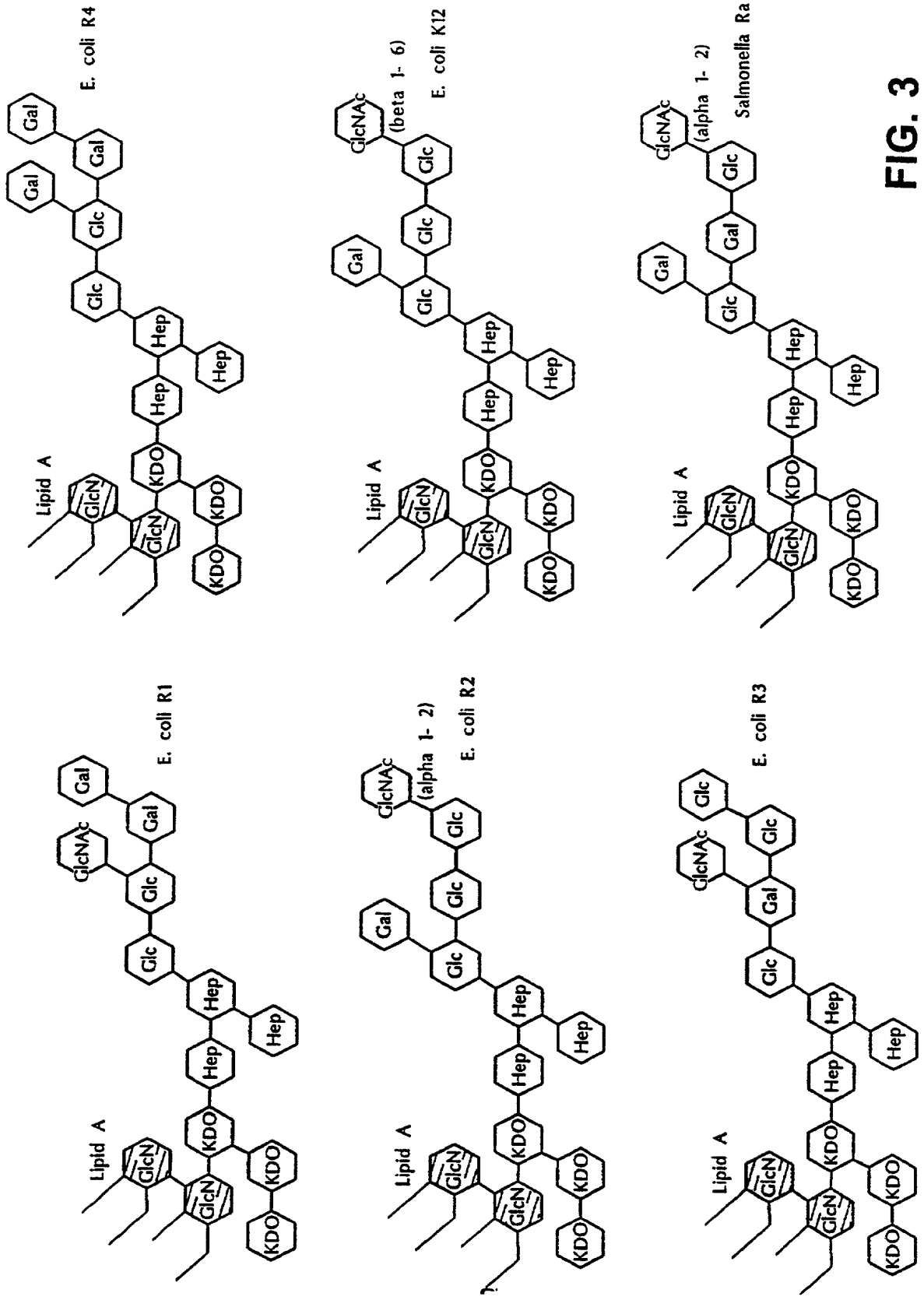
E. coli R4
Gal
Gal
Gal
Glc
Glc
Hep
Hep
Hep
KDO
KDO
KDO
Lipid A
GlcN
GlcN
E. coli K12
GlcNAc
(beta 1- 6)
Glc
Gal
Glc
Glc
Hep
Hep
Hep
KDO
KDO
KDO
Lipid A
GlcN
GlcN
Salmonella Ra
GlcNAc
(alpha 1- 2)
Glc
Gal
Glc
Hep
Hep
Hep
KDO
KDO
KDO
Lipid A
GlcN
GlcN
E. coli R1
Gal
Gal
GlcNAc
Glc
Glc
Hep
Hep
Hep
KDO
KDO
KDO
Lipid A
GlcN
GlcN
E. coli R2
GlcNAc
(alpha 1- 2)
Glc
Gal
Glc
Glc
Hep
Hep
Hep
KDO
KDO
KDO
Lipid A
GlcN
GlcN
E. coli R3
Glc
Glc
GlcNAc
Gal
Glc
Hep
Hep
Hep
KDO
KDO
KDO
Lipid A
GlcN
GlcN

FIG. 3

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COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled VACCINE AGAINST LIPOPOLYSACCHARIDE CORE, the specification of which:

[] is attached hereto.
[X] was filed on November 12, 1999 as Application Serial No. 09/423,546 and was amended on ________________.
[] was described and claimed in PCT International Application No. _______________ filed on _______________ and as amended under PCT Article 19 on _______________.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §119(e)(1) of any United States provisional application(s) listed below:

U.S. Serial No.	Filing Date	Status

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

U.S. Serial No.	Filing Date	Status

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Application No.	Filing Date	Priority Claimed
PCT	WO98/51217	5/15/98	[X] Yes [] No
			[] Yes [] No